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14. ABSTRACT

An anti-cancer strategy has been developed that relies on a synthetic ligand to 1) specifically target the cell surface, 2)recruit antibodies to the tumor cell, and 3) subsequently initiate a cytotoxic immune response. This small-molecule is composed of two distinct motifs: an RGD peptomimetric that binds with high affinity to the alpha (v) beta(3) integrin (a cell-surface marker found to be upregulated in numerous cancers) and the alpha-Gal trisaccharide antigen that interacts with endogenous human antibody. Using out bifunctional conjugate, we found that the multivalent properties of anti -Gal binding can be exploited to triger complement-mediated lysis of only those cells that display elevated levels of alpha (v) beta (3) integrin. The ability of anti-Gal to cause complement-medicated lysis depends on the concentration of alpha-Gal epitopes displayed on teh cell surface.

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integrins, alpha-Gal, antibody, immunotherapy

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INTRODUCTION

The Nobel Laureate (1908) Paul Ehrlich is perhaps best known for his visionary description of the “magic bullet”, a substance able to seek out and kill disease-causing cells while leaving normal ones unaffected. The magic bullet concept is the inspiration for this project. While there have been major advances in cancer chemotherapy, agents that specifically attack cancer over normal cells have been elusive. We sought to address this deficiency by developing a tumor targeting strategy with exquisite selectivity. We postulated that we could recruit natural cell targeting and killing mechanisms to distinguish between tumor and normal cells. Our strategy involves using small molecules with two binding epitopes (termed “bifunctional conjugates”) that have the following two properties: one epitope binds with high affinity to a receptor on the cancer cell surface; the other can bind weakly but simultaneously to a naturally occurring human antibody. When multiple copies of the bifunctional ligand coat the cell, antibodies are recruited to the tumor. Complement proteins then bind the antibody-coated tumor cells and mediate cytotoxicity. Multivalent interactions are required for antibody binding and complement recruitment; therefore, the antibody will bind tightly only to cells that are decorated with many copies of the bifunctional conjugate. As a result, tumor cells expressing high levels of the specific cell-surface receptor are destroyed but not normal cells. We have demonstrated that this strategy is highly effective and selective. These results have been presented at numerous scientific meetings and are documented in 2 peer-reviewed publications. The results from this project are serving as a blueprint for developing new classes of bifunctional ligands and for implementing and testing our strategy in pre-clinical and translational studies.

BODY

The overall objective of this research program was to develop a new class of anti-tumor agents and evaluate its selectivity for tumor and normal cells. Task 1 was focused on generating a prototype anti-tumor agent and task 2 was focused on testing it. Both of these tasks were accomplished, as described in the following sections.

A key initial goal, as outlined in *Task 1*, was to chemically synthesize bifunctional conjugates that could serve as anti-cancer agents. The compounds consist of a high-affinity cancer cell-targeting agent linked to the low-affinity immunogenic carbohydrate epitope known as alpha-Gal. We identified a tight-binding peptidomimetic ligand (an RGD analog) for the alpha(v) beta(3) ($\alpha_v\beta_3$) integrin, assembled the carbohydrate with a linker for attachment, and developed a strategy to conjugate these two motifs together (Compound **1**, Figure 1). Our route is modular and therefore can be used to connect any cell-surface targeting agent to the alpha-Gal oligosaccharide (or any other functional epitope). In the course of our studies, we have exploited the modularity of our synthetic approach to generate a fluorescent integrin ligand and a conjugate of the integrin ligand to the anti-cancer agent doxorubicin (vide infra).

With our bifunctional ligand in hand, we examined its ability bind relevant target proteins: the $\alpha_v\beta_3$ integrin and the anti-Gal antibody (*Task 2*). We also evaluated the ability of this bifunctional compound to bind to cancer cells that display the $\alpha_v\beta_3$ integrin. The first objective was accomplished using an assay assessing the ability of a compound to inhibit binding of $\alpha_v\beta_3$ integrin-producing cells to vitronectin (1). Vitronectin is an extracellular matrix protein that binds tightly to integrins, and it is an excellent substrate for testing competitor integrin ligands (2). The peptidomimetic ligand alone and the bifunctional ligand both are potent cell adhesion inhibitors, indicating that they are excellent integrin ligands. These results are described in our publication in *ChemBiochem*(1). We also have appended a fluorophore to the RGD analog to generate peptidomimetic derivative **2**. Fluorescence microscopy and flow cytometry experiments indicate that this probe interacts with cells displaying the $\alpha_v\beta_3$ integrin but not those that do not (see (3) and appendix). Thus, the bifunctional probe binds to the target cell surface receptor.

To determine whether the alpha-Gal epitope within the bifunctional ligand can interact with anti-Gal antibodies, we used flow cytometry. We exposed cells displaying the $\alpha_v\beta_3$ integrin to the bifunctional ligand and tested whether the alpha-Gal epitope could be detected. To this end, we employed flow cytometry to monitor anti-Gal binding. The presence of anti-Gal could be detected under these conditions (3). These results indicate that both epitopes, the integrin binding and the anti-Gal binding moieties, are functional.

With support that the designed conjugate **1** could bind the requisite proteins, we tested whether it could recruit sufficient anti-Gal to a tumor cell surface to mediate cell lysis. To this end, we exposed this ligand to a cell line (WM115) that displays high levels of $\alpha_v\beta_3$ integrin. We then added human serum, which contains both anti-Gal antibodies and complement proteins. If the bifunctional ligand can recruit anti-Gal to the cell surface, this mixture should effect cell lysis. Using a fluorescence-based complement-mediated cytotoxicity assay (as proposed in *Task 3*), we assessed the ability of the bifunctional conjugate to promote tumor cell killing. Compound **1** was effective (Carlson et al., *ACS Chem. Biol.* **2007**, *2*, 347–355, see attached). These results indicate that the designed bifunctional ligand can recruit endogenous antibodies (anti-Gal) and complement to kill tumor cells.

Our strategy was designed to be highly specfic. We envisioned that our approach could discriminate not only between cells with and without the target receptor, but also between cells with the low and high levels of the target. To test this hypothesis that the amount of cell surface $\alpha_v\beta_3$ integrin influences cell killing, we needed a panel of various tumor cell lines presenting different levels. As proposed in *Task 2*, we used fluorescein-labeled integrin ligand **3** to assess these levels. When these different cell lines were tested in our cytoxocity assay, tumor cells with high levels of the cell-surface integrin were killed, but “normal” cells displaying low levels were not (Carlson et al., *ACS Chem. Biol.* **2007**, *2*, 347–355, see attached). These results underscore the high selectivity of using multivalent interactions to discriminate selectively between cells.

We wanted to compare the cell type selectivity we achieved with our multivalent targeting to that obtained with a conventional targeted chemotherapeutic. Accordingly, we synthesized a new conjugate in which the well-known chemotherapeutic agent doxorubicin was appended to our

tumor-homing agent (Figure 1, compound **2**). We tested this compound in a cellular cytotoxocity assay to compare the cell killing selectivities of compounds **1** and **2**. The traditional targeting agent **2**, killed all the cells displaying the $\alpha_v\beta_3$ integrin, regardless of its cell-surface concentration (Figure 2). In contrast, compound **1** exhibited high selectivity. These results highlight the specificity of our strategy.

We postulated that our bifunctional conjugate achieves cell killing through multivalent binding—both anti-Gal binding and complement-mediated cell killing involve multivalent interactions. If cell killing depends on multivalent interactions, cell killing should be highly sensitive to ligand concentration. Thus, we examined the percentage of cell killing relative to bifunctional conjugate concentration. The concentration of ligand **1** has a dramatic effect on its ability to mediate cell killing (Figure 3). In contrast, there is a much more gradual concentration dependence for doxorubicin conjugate **2**. These data support the mechanistic hypothesis underlying our cancer cell targeting approach.

With these proof-of-principle results, we are pursuing an in vivo assessment of our bifunctional conjugate-targeting strategy. Our plan was to test conjugate **2** in a mouse xenograft model. One complicating feature of conducting an in vivo test is that mice display the alpha-Gal epitope on their cell surfaces; consequently, unlike humans they do not produce anti-Gal antibodies. An alpha-Gal knockout (KO) mouse has been generated (4), however, by deleting the glycosyltransferase that generates the alpha-Gal epitope. This strain has been graciously provided to us by Dr. Galili.

A second requirement for in vivo testing is to implant xenografts that are both $\alpha_v\beta_3$ integrin-positive and alpha-Gal negative. To this end, we tested a variety of murine cancer cell lines for the latter attribute. Using an enzyme-linked immunosorbent assay (Figure 4), we identified that B16F10 cell line as lacking the alpha-Gal epitope. Moreover, several examples in the literature used this cell line in a xenograft model. We next examined whether this cell line displays $\alpha_v\beta_3$ integrin. We employed both the RGD-fluorescein probe and antibodies directed against the heterodimeric receptor. These experiments indicated that, while $\alpha_v\beta_3$ appears to be present, its cell surface concentration is low (data not shown). Given this result, we are currently

investigating methods to increase the number of integrin receptors (i.e., transfection) and examining other cell types that might have both of the key characteristics required to test our strategy in vivo.

A third concern is that our subsequent studies of cell killing have indicated that the binding of anti-Gal to cells treated with the bifunctional ligand is not as a robust as is desired for in vivo studies (i.e., the conditions used in the cell studies are not readily adaptable to in vivo targeting). Therefore, we are altering the structure of the bifunctional ligand to optimize its efficacy. Specifically, we are generating bifunctional ligands with longer linkers separating the integrin- and anti-Gal-binding epitopes. We envision that increasing linker length will render the α-Gal epitope more accessible upon integrin binding. In addition, we have devised ligands that display up to 4 copies of the α-Gal epitope, and these should bind with higher avidity to anti-Gal. Lastly, we envision targeting two types of cell surface receptors, such that the total concentration of α-Gal displayed on the cell surface is increased. We are in the process of pursuing these experiments in a collaboration with Dr. Paul Sondel.

KEY RESEARCH ACCOMPLISHMENTS

- Produced a functionalized RGD peptidomimetic ligand with functional groups suited for bioconjugation reactions. This compound maintains high affinity and excellent selectivity for the $\alpha_v\beta_3$ integrin.
- Assembled a bifunctional conjugate consisting of an immunogenic trisaccharide (alpha-Gal) and an RGD mimetic (compound **1**).
- Demonstrated that bifunctional ligand **1** binds selectively to cells presenting the $\alpha_v\beta_3$ integrin.
- Detected simultaneous binding of compound **1** to both the $\alpha_v\beta_3$ integrin and the anti-Gal antibody.
- Developed a fluorescence-based assay for complement-dependent cytotoxicity.
- Showed that the bifunctional ligand **1** can promote anti-Gal recruitment to tumor cells and subsequent complement-mediated killing of tumor cells.
- For comparing monovalent and multivalent cell targeting, generated a conjugate anti- consisting of the toxin doxorubicin and the integrin ligand (compound **2**).
- Used compound **3** to identify a panel of tumor cell lines expressing different levels of the $\alpha_v\beta_3$integrin.
- Showed that compound
- Demonstrated that bifunctional ligand **1** promotes selective cell killing. Only those cells with high levels of surface integrin are destroyed. We also showed that this cytotoxicity profile is dramatically different than that of traditional targeted anti-cancer agents, such as **2**.

REPORTABLE OUTCOMES

Peer-reviewed manuscripts

1. Owen, R.M., Carlson, C.B., Xu, J., Mowery, P., Fasella, E., Kiessling, L.L. "Bifunctional ligands that target cells displaying the alpha(v) beta(3) integrin" *ChemBioChem* **2007** *8* (1), 68–82.
2. Carlson, C.B., Mowery, P., Owen, R.M., Dykhuizen, E.C., Kiessling, L.L. "Selective tumor cell targeting using low-affinity, multivalent interactions" *ACS Chem. Biol*. **2007** 2 (2), 119–127.

News and attention from scientific community

The 2007 paper published in *ACS Chemical Biology* was highlighted in various places around the web.

3. Our research article was featured on the cover of the Feb 2007 issue of the journal.http://pubs3.acs.org/acs/journals/toc.page?incoden=acbcct&involume=2&inissue=2
4. News highlight in *Chemical and Engineering News*, "Strength in numbers" written by Celia Henry Arnaud. http://pubs.acs.org/cen/news/85/i08/8508notw6.html

Poster / Oral presentations

5. C.B. Carlson, R.M. Owen, P. Mowery, J.A. Hank, P.M. Sondel, L.L. Kiessling. "Bifunctional immunotherapeutic agents for the treatment of cancer." Abstracts of Papers, 228th ACS National Meeting, Philadelphia, PA (2004).
6. C.B. Carlson, R.M. Owen, P. Mowery, E.C. Dykhuizen, L.L. Kiessling. "Bifunctional ligands for selective cell targeting via multivalent interactions". AACR-NCI-EORTC International Conference, Philadelphia, PA (2005).

Funding applied for based on work supported by this award

Based on the progress from this award, a translational science project has been funded

7. Dept. of Defense Ovarian Cancer Research Program Application, Translational Research Partnership "*Ovarian Cancer Immunotherapy Using Redirected Endogenous Anti-Gal Antibody***".**

Patents, licenses, degrees, cell line development, tissue or serum repositories, etc.

N/A

CONCLUSION

This project has allowed us to blend various area of science (e.g., xenotransplantation, synthetic organic chemistry, immunology, and the integrin family of receptors) to tell a complete story that highlights molecular recognition on various levels. Additionally, our basic research has produced a lead compound that we hope to carry through further to pre-clinical and translational studies.

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1. Owen RM, *et al.* (2007) Bifunctional ligands that target cells displaying the alpha(v)beta(3) integrin. *ChemBioChem* 8(1):68-82.

2. Plow EF, Haas TK, Zhang L, Loftus J, & Smith JW (2000) Ligand binding to integrins. *J. Biol. Chem.* 275(29):21785-21788.

3. Carlson CB, Mowery P, Owen RM, Dykhuizen EC, & Kiessling LL (2007) Selective tumor cell targeting using low-affinity, multivalent interactions. *ACS Chem. Biol.* 2(2):119-127.

4. Tearle RG, *et al.* (1996) The alpha-1,3-galactosyltransferase knockout mouse - Implications for xenotransplantation. *Transplantation* 61(1):13-19.

APPENDICES

Please refer to the *ChemBioChem* and *ACS Chemical Biology* journal articles below.

Carlson CB, Mowery P, Owen RM, Dykhuizen EC, Kiessling LL. (2007) Selective tumor cell targeting using low-affinity, multivalent interactions. ACS Chem Biol. 2(2):119-27. PMID: 17291050

Owen, R.M., Carlson, C.B., Xu, J., Mowery, P., Fasella, E., and Kiessling, L.L. (2007) Bifunctional Ligands that Target Cells Displaying the alpha(v) beta (3)Integrin, ChemBioChem, 8, 68–82.

SUPPORTING DATA

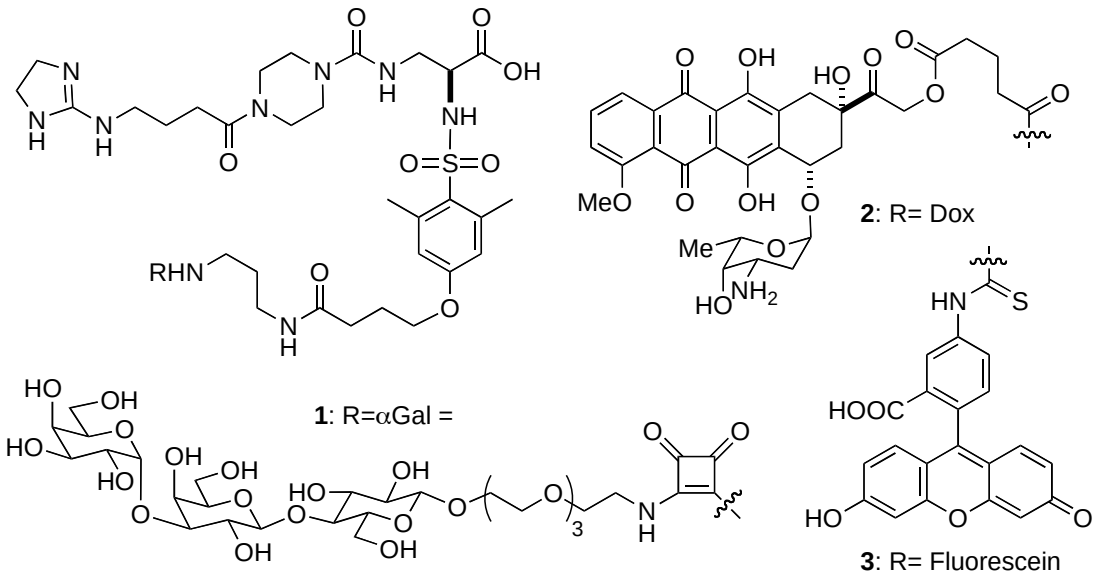

Figure 1. Structures of bifunctional molecules used in the reported anti-cancer studies. Compound **1**, which consists of an alpha(v) beta(3) integrin ligand linked to the alpha-Gal trisaccharide, was designed to recruit anti-Gal antibodies to tumor cells, via multivalent bindings. This recruitment should facilitate complement-mediated lysis. Bifunctional conjugate **2** is composed of the integrin ligand linked to doxorubicin (Dox), an anti-cancer agent used in the clinic; its ability to kill tumor cells does not depend on multivalent binding. Attachment of a fluorophore to the integrin ligand generates fluorescent probe **3**, which was employed in microscopy and flow cytometry.

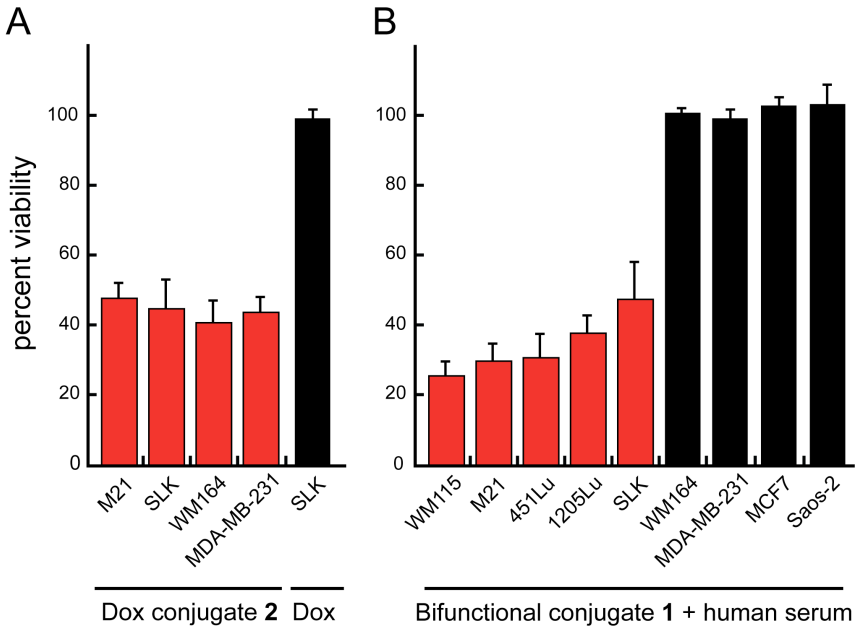

Figure 2. Bifunctional conjugate **1** mediates selective cell killing. a) Four cell lines were treated with compound **1** and their cell viability was assessed using a standard tetrazolium salt-based assay. Treatment with the DOX conjugate resulted in >50% cell death, irrespective of the levels of $\alpha_v\beta_3$ integrin (red). Unmodified DOX (25 nM) had no effect on the cells (black); b) Data from all nine cell lines tested in the complement-dependent cytotoxicity assay. The cell lines that were lysed

efficiently following treatment with bifunctional ligand **1** (10 nM) and human serum are shown in red; those that were unaffected are depicted in black.

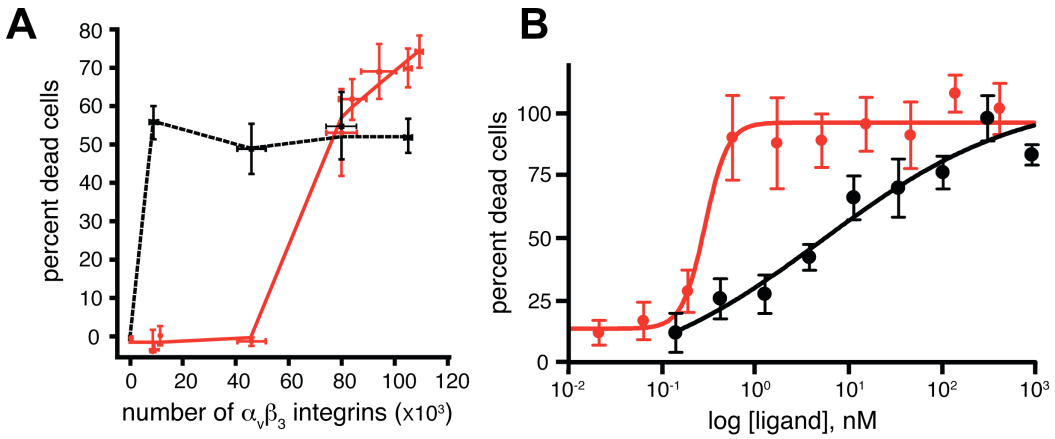

Figure 3. Features of cell recognition by multivalent interactions. A: The number of $\alpha_v\beta_3$ integrin receptors available for binding is plotted against the percentage of dead cells (data from Figure 2). A minimum number of cell-surface target receptors is required to generate a functional multivalent interaction with anti-Gal antibodies following treatment with bifunctional conjugate **1**. The dashed black curve denotes the activity of the DOX conjugate **2**. It kills cells displaying high and low levels of the target receptor. Conversely, cells with a low concentration of integrin receptor are unaffected by α-Gal-mediated cytotoxicity, as shown by the solid red curve. B. Dose response curves for compounds **2** (Dox conjugate, black) or **1** (bifunctional ligand, red). There is marked concentration dependence for the ability of compound **1** to effect cell death, which is indicative of a process involving cooperative multivalent interactions. The gradual dependence on concentration for the doxorubicin conjugate **2** is typical of a process that involves monovalent interactions.

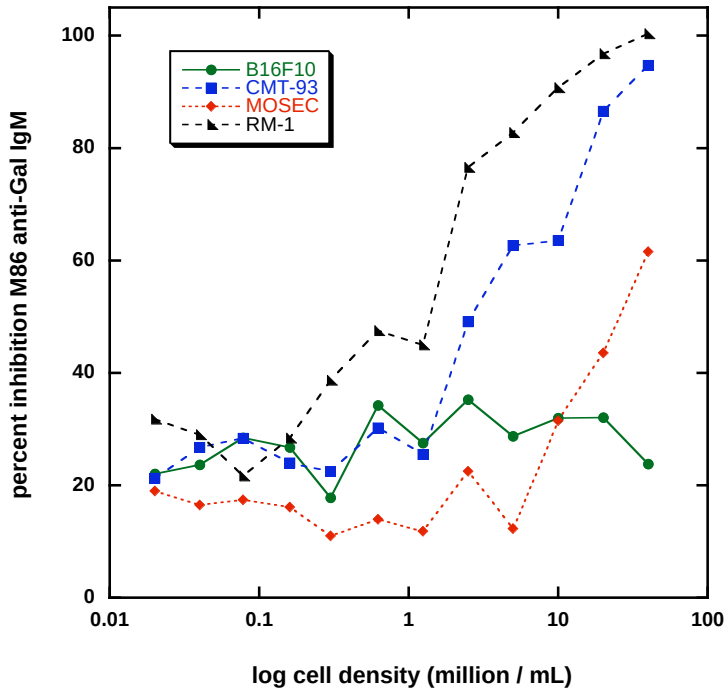

Figure 2. ELISA to test for alpha-Gal epitopes on the cell surface.

FINAL REPORT BIBLIOGRAPHY

All final reports must include a bibliography of all publications and meeting abstracts and a list of personnel (not salaries) receiving pay from the research effort.

Owen, R.M., Carlson, C.B., Xu, J., Mowery, P., Fasella, E., Kiessling, L.L. "Bifunctional ligands that target cells displaying the alpha(v) beta(3) integrin" *ChemBioChem* **2007** *8* (1), 68–82.

Carlson, C.B., Mowery, P., Owen, R.M., Dykhuizen, E.C., Kiessling, L.L. "Selective tumor cell targeting using low-affinity, multivalent interactions" *ACS Chem. Biol*. **2007** 2 (2), 119–127.

C.B. Carlson, R.M. Owen, P. Mowery, J.A. Hank, P.M. Sondel, L.L. Kiessling. "Bifunctional immunotherapeutic agents for the treatment of cancer." Abstracts of Papers, 228th ACS National Meeting, Philadelphia, PA (2004).

C.B. Carlson, R.M. Owen, P. Mowery, E.C. Dykhuizen, L.L. Kiessling. "Bifunctional ligands for selective cell targeting via multivalent interactions". AACR-NCI-EORTC International Conference, Philadelphia, PA (2005).

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